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The Inactivation of α-Chymotrypsin with Methyl-, Trideuteriomethyl-, and Trifluoromethyl-Substituted N-Phenyl-α-bromoacetamides

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Abstract: The kinetics of the irreversible reaction of *o*-, *m*-, and *p*-methyl- and -trifluoromethyl-substituted N-phenyl-α-bromoacetamides with α-chymotrypsin have been studied at pH 6.0. Dissociation constants for the initially formed enzyme–inhibitor complexes have been determined or estimated and rate constants for reaction of these complexes to give inhibited proteins have been obtained. The *para* position seems to be most sensitive to substitution; a methyl group at this position enhances the stability of the enzyme–inhibitor complex but does not affect the rate of subsequent reaction while a *p*-trifluoromethyl group at this position greatly reduces both binding and this reaction rate. The behavior of the *p*-trideuteriomethyl-substituted inhibitor is indistinguishable from that of the protio compound.

An important adjunct to the traditional use of reaction kinetics for the study of enzymic active sites is the chemical modification of amino acids in the enzyme. With the ability to alter specific amino acid residues, often near the active center of the protein, one has a potentially powerful tool for the study of local environments in the enzyme, especially if the modification affords a protein with physical properties amenable to study by spectroscopic techniques. The use of "reporter groups" for the exploration of the active site of α-chymotrypsin is rather well developed.[2-6] Although some use has been made of proton magnetic resonance spectroscopy to investigate this enzyme,[7-9] one cannot expect a great deal of success in the reporter group type of study by this technique since the proton signals of the reporter group will be obscured by the large number of proton resonances of the enzyme itself. We have, therefore, embarked upon the preparation of modified α-chymotrypsins which contain fluorine or deuterium in the reporter group, with the hope that nmr studies of these labeled materials will provide new information about the properties of this enzyme.

Methionine-192 has been a favorite target for investigations which utilize the reporter group idea because of its high reactivity toward "active" halogens. Lawson and Schramm have investigated the irreversible inhibition of α-chymotrypsin by a series of N-substituted α-bromoacetamides as well as α-bromoacetophenone and benzyl bromide.[10] The results of these workers, and others,[2,3,11,12] show that organic molecules with an activated halogen (usually bromine) and a large nonpolar group (often aromatic) will inactivate this enzyme irreversibly by first forming a complex with the active site followed by displacement of the halogen by the sulfur atom of methionine-192. Although the compounds investigated to date inactivate the enzyme at widely different rates, it is usually the methionine-192 residue which is attacked. It has been suggested that this residue is near a "hydrophobic" binding locus;[13] a properly constituted inhibitor molecule may occupy this site *via* a hydrophobic interaction which places the α-bromoacetyl moiety of this species into the correct orientation for reaction with the thio ether linkage of the methionine.

With these observations in mind we prepared a series of methyl and trifluoromethyl substituted N-phenyl-α-bromoacetamides (Ia–h) and have studied their ability to irreversibly inhibit α-chymotrypsin.

(1) NDEA Title IV Fellow, 1966–1969. This work will constitute a portion of the Ph.D. Thesis to be submitted by E. W. B.

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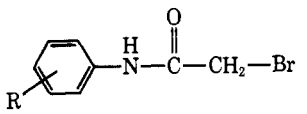

Ia, R = H
b, R = *o*-CH_3
c, R = *m*-CH_3
d, R = *p*-CH_3
e, R = *p*-CD_3
f, R = *o*-CF_3
g, R = *m*-CF_3
h, R = *p*-CF_3

Results

Presumably the reaction of all of the bromoacetanilides with this enzyme involves the formation of a reversible enzyme–inhibitor complex, symbolized EI, followed by an essentially irreversible displacement of bromine by the nucleophilic thio ether group on the enzyme.[10] The reaction can be represented by the simple kinetic scheme shown in eq 1. We have used the symbol $\widehat{EI}$ to denote the catalytically inactive enzyme which results from subsequent, internal reaction of the EI complex and we shall presume that the rates involved in the equilibration step are rapid enough to maintain an equilibrium concentration of EI.[14] The equilibrium constant, K_I, shall be written as a dissociation constant. The total concentration of potentially active enzyme, E_t, at any time is given by $E_t = E + EI$. Assuming that once the enzyme has been irreversibly inactivated it can no longer participate in any important way in eq 1, it follows that the appearance of irreversibly inactivated enzyme ($\widehat{EI}$) or the disappearance of active enzyme (E or EI) will be a process which is first order with respect to total active enzyme concentration and which is characterized by a rate constant

$$E + I \underset{K_I}{\rightleftharpoons} EI \xrightarrow{k_3} \widehat{EI} \qquad (1)$$

$$\frac{d[\widehat{EI}]}{dt} = \frac{k_3[E_t]}{1 + K_I/[I]} \qquad (2)$$

$$k_{obsd} = \frac{k_3}{1 + K_I/[I]} \qquad (3)$$

if the concentration of the inhibitor, I, is large enough that it does not change significantly during the reaction. If solubility permitted, one could run the inactivation under conditions such that $[I] \gg K_I$ and k_3 could be measured directly. This is not often true, so that the observed rate of enzyme inactivation usually depends both on the binding constant, K_I, and the rate constant, k_3.

We have determined k_{obsd} for the inhibitors Ia–h using a 20-fold excess of inhibitor over the initial enzyme concentration. Aliquots of the reaction mixture were taken at regular intervals and the concentration of the remaining active enzyme (E_t) was determined by an assay based on the hydrolysis of N-glutarylphenylalanine *p*-nitroanilide (GPNA).[15] Plots of log $[E_t]$ *vs.* time were linear to at least 90% reaction; the resulting rate constants (k_{obsd}) are collected in Table I. Enzyme activity was essentially completely lost during these reactions.

(14) Nmr evidence suggests that this condition is likely fulfilled; B. D. Sykes, *J. Amer. Chem. Soc.*, 91, 949 (1969).

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Table I. Inactivation of α-Chymotrypsin by Substituted N-Phenyl-α-bromoacetamides at 27.5°[a]

Substituent	$[E]_0$,[b] mg/ml	$[I_0]$,[b] m*M*	Cosolvent[c]	$k_{obsd} \times 10^4$, sec^{-1}
H	1.8	1.29	Ethanol	3.42
	3.6	3.58	Ethanol	5.27
o-CH_3	1.8	1.29	Ethanol	0.28
m-CH_3	1.8	1.29	Ethanol	3.50
p-CH_3	1.8	1.29	Ethanol	6.6[d]
	2.5	1.82	DMSO	7.80
p-CD_3	2.5	1.82	DMSO	7.85
o-CF_3	1.8	1.29	Ethanol	0.035
	2.5	1.82	DMSO	0.052
m-CF_3	1.8	1.29	Ethanol	0.38
	2.5	1.82	DMSO	0.55
p-CF_3	1.8	1.29	Ethanol	0.086

[a] 0.1 *M* phosphate buffer at pH 6.0. The rate constants reported are averages of two or more separate determinations. [b] Initial concentrations of enzyme and inhibitor, respectively. [c] Solvent was a mixture of 10 vol % of the cosolvent and water. [d] One determination.

The data indicate that inactivation of α-chymotrypsin by N-phenyl-α-bromoacetamides is quite sensitive to the nature and orientation of the substituent on the phenyl ring, but in order to interpret these results it was necessary to determine independently the binding constants, K_I. This determination was made by conventional competitive-inhibition kinetic experiments, again using GPNA as substrate under conditions where the rate of $\widehat{EI}$ formation was negligible.[16] The equilibrium constants so obtained are recorded in Table II. All of the inhibitors showed competitive inhibition except If and Ih which were too insoluble to permit accurate measurements. Assuming competitive inhibition by the two insoluble compounds, it was possible to estimate a lower limit for K_I for these materials by the use of eq 4, where V is the velocity of the enzymatically catalyzed hydrolysis reaction of GPNA in the absence of inhibitor,

Table II. Equilibrium and Rate Constants for the Inactivation of α-Chymotrypsin by Substituted N-Phenyl-α-bromoacetamides[a]

Substituent	Cosolvent	K_I, m*M*[b]	$k_3 \times 10^3$, sec^{-1}
H	Ethanol	4.2	1.3
o-CH_3	Ethanol	7.3	0.19
m-CH_3	Ethanol	3.0	1.1
p-CH_3	Ethanol	0.88	1.1
	DMSO	(0.88)[d]	1.2
p-CD_3	DMSO	(0.88)[c]	1.2
o-CF_3	Ethanol	~30[e]	0.07
	DMSO	(~30)[d]	0.08
m-CF_3	Ethanol	7.8	0.26
	DMSO	(7.8)[d]	0.29
p-CF_3	Ethanol	~10[e]	0.08

[a] Ethanol was used as a cosolvent in all binding constant determinations. The buffer was 0.05 *M* Tris at pH 7.45. [b] These values are reliable to about ±10%. [c] Assumed to have the same K_I as the *p*-CH_3 compound. [d] Assumed to have the same K_I as when ethanol is the cosolvent. [e] Estimated as discussed in the text.

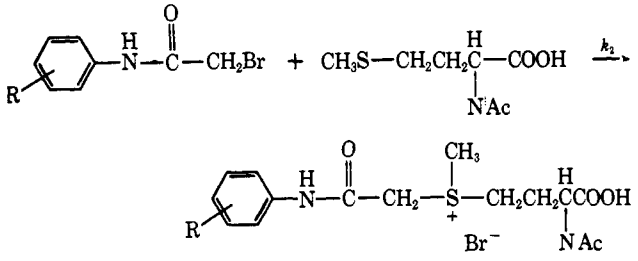
$$\frac{V}{V_I} = 1 + \frac{[I]}{K_I}\left(\frac{K_M}{K_M + [S]}\right) \qquad (4)$$

V_I is the velocity at inhibitor concentration [I], K_M is the

(16) M. Dixon and E. C. Webb, "Enzymes," Academic Press, New York, N. Y., 1958, p 180.

Michaelis–Menton constant for GPNA, and [S] is the concentration of GPNA.[17] These estimates are included in Table II. With the availability of the binding constant information, one could then compute the rate constants, k_3, by means of eq 4. These quantities are also listed in Table II.

The variations in the rate constant, k_3, could merely be a reflection of the traditional steric or electronic effects of the substituents on the phenyl ring. In order to appraise the importance of these effects, the kinetics of a model reaction between the various bromoacetamides and N-acetylmethionine were determined by



monitoring the evolution of bromide ion. This reaction was shown to be second order, first order in each reactant. To obtain the rate constants reported in Table III, the reaction was run under pseudo-first-order conditions with the N-acetylmethionine in 100-fold molar excess over the N-phenyl-α-bromoacetamide. As can be seen from Table III, the rate constants for these model reactions are nearly identical regardless of the substituent except when the substituent is in the *ortho* position. In these latter cases, the model reaction is slowed by about a factor of two.

Table III. Rate Constants for the Reaction of N-Acetylmethionine with Substituted N-Phenyl-α-bromoacetamides at 25°[a]

Substituent	$k_2 \times 10^3$, M^{-1} sec^{-1} [b]
H	1.3
o-CH_3	0.64
m-CH_3	1.3
p-CH_3	1.2
p-CD_3	1.2
o-CF_3	0.64
m-CF_3	1.1
p-CF_3	1.3

[a] pH 6.0 in 10% ethanol–water. [b] Reproducibility in replicate runs was ±10%.

Discussion

The rates of enzyme inactivation do not appear to be very sensitive to hydrogen ion concentration. For the reaction of the unsubstituted compound we observe the same rate at pH 6.0 as was reported by Schramm and Lawson at pH 5.0.[10] Similarly, the rate of inactivation by the *o*-methyl- and *o*-trifluoromethyl-substituted materials are the same at pH 4.0 as at pH 6.0. It is usually found that the dissociation constants, K_I, for reversible inhibitors are insensitive to pH in the range 6–8.[18] It seems, therefore, permissible to mix the rate and dissociation constant data reported above even though they were obtained at slightly different hydrogen ion concentrations. There also appears to be no large effect of cosolvent since the results obtained are nearly identical when either ethanol or dimethyl sulfoxide is used in this capacity.

Throughout this work we have made the tacit assumption that the methionine-192 residue of α-chymotrypsin is indeed being modified by reaction with the α-bromoacetamides. This assumption seems reasonably based on precedent,[2,3,10] and will be assumed to be correct in the discussion which follows.

Most simply interpreted, the fact that the EI complexes of the unsubstituted and *m*- and *p*-methyl-substituted N-phenyl-α-bromoacetamides react at nearly the same rate suggests that the relative orientation of the reacting moiety in each of these reactions is very similar. The reduced reactivity of the *o*-methyl-substituted compound is in line with the observation made with the model reaction system. The major effect of methyl substitution in this system is to modify the binding constant, K_I. Alkyl substitution in the portion of a reversible enzyme inhibitor which utilizes the hydrophobic mechanism for binding usually leads to a stronger enzyme–inhibitor interaction and, therefore, a smaller K_I.[19] Methyl groups at the *ortho* and *meta* positions do not greatly effect the binding interaction but a *p*-methyl group results in fivefold better binding (as measured by K_I) and, as a result, N-(*p*-tolyl)-α-bromoacetamide is by far the most efficient inhibitor of α-chymotrypsin encountered in this study.

As is apparent from Table II, the change of a methyl substituent to a trifluoromethyl substituent has a profound effect on both the rate constant for inactivation (k_3) and the dissociation constant (K_I) for the enzyme–inhibitor complex, EI. The ratios of k_3 (and K_I) for the methyl-substituted compound to k_3 (and K_I) for the corresponding trifluoromethyl compound are displayed in Table IV. Although any quantitative discussions of these ratios must depend on the exact values of K_I for the *o*- and *p*-trifluoromethyl-substituted compounds, it does seem clear that rate constants for reaction of the EI complexes are decreased by roughly the same relative amount when the group in either the *ortho* or *meta* ring positions are compared. Likewise, trifluoromethylation at either of these positions tends to decrease the binding ability of the inhibitor about the same amount. Whatever the origins of the effect of replacing methyl by trifluoromethyl, they seem to operate in about the same manner at the *ortho* and *meta* positions of the inhibitor.

Table IV. Comparison of the Rate and Equilibrium Constants for Methyl- and Trifluoromethyl-Substituted Inhibitors

Substituent position	k_2, CH_3/ k_2, CF_3[a]	k_3, CH_3/ k_3, CF_3[b]	K_I, CH_3/ K_I, CF_3[c]
ortho	1.0	2.7	0.25
meta	1.1	4.0	0.38
para	0.89	15.0	0.09

[a] Ratio of the rate constants for model reaction, *cf.* Table III. [b] Ratio of the rate constants, k_3, for the enzyme inhibition, *cf.* Table II. [c] Ratio of the dissociation constants, K_I, for the enzyme inhibition, *cf.* Table II.

The *para*-substituted N-phenyl-α-bromoacetamides are much more responsive to the exchange of a methyl

(17) E. A. Dawes, "Quantitative Problems in Biochemistry," Williams and Wilkins, Baltimore, Md., 1963, p 135.
(18) F. J. Kezdy and M. L. Bender, *Ann. Rev. Biochem.*, **34**, 49 (1965).
(19) Reference 13, p 53.

for a trifluoromethyl group.[20] The rate constant for reaction of the *p*-methyl EI complex is more than 15 times larger than when a trifluoromethyl group occupies this position. That the binding of the *p*-methyl compound to the enzyme is about ten times more effective than the binding of the *p*-trifluoromethyl inhibitor provides an additional indication of the sensitivity of this inhibition reaction to the local characteristics of the terminus of the N-phenyl portion of these irreversible inhibitors. Our experiments have not been sensitive enough to detect any difference in the behavior of the methyl and trideuteriomethyl group at this position, however.

The effects of fluorine substitution in biochemical systems have been discussed recently[21] but the properties of the trifluoromethyl group which are responsible for the variations in kinetic and equilibrium parameters described above are obscure. Presumably the spatial arrangement of atoms in the enzyme–inhibitor complex is a rather specialized one and a part of the changes observed in this work may be a result of a steric effect. The steric requirement of a methyl *vs.* a trifluoromethyl group is difficult to evaluate in systems such as the one described here. It is believed that trifluoromethyl is slightly larger than methyl in the cyclohexane system;[22] a calculation of the effective radius of the trifluoromethyl group using covalent and van der Waal's radii[23] suggests that the volume of this group is about twice that of a methyl group.[24] The utility of this calculation is befogged by solvent effects. Dipole moment studies indicate that benzotrifluoride has a much greater dipole moment than toluene[25] so that the more polar CF_3 group will likely be highly solvated, rendering its effective size relative to methyl considerably different from the value computed above. Alternatively, dispersion forces of an attractive nature may account for our observations. It is clear from these results that α-chymotrypsin can readily distinguish between a proton and a fluorine atom; it is not yet apparent how this distinction is made. We hope that nmr experiments with the deuterated and fluorinated enzyme described herein will help elucidate this problem.

Experimental Section

Materials. ***p*-Trideuteriomethylbenzoic acid** was prepared by equilibrating a 2.5 *M* solution of the potassium salt in deuterium oxide solution at 180° in a sealed bomb.[26] The exchange was followed by nmr analysis of aliquots taken as the reaction progressed. After 80% conversion, the solvent was evaporated and fresh D_2O added to the residue. The exchange reaction was essentially complete at 96 hr as evidenced by nmr. The yield of deuterated acid was 59%; mass spectral analysis showed 91% CD_3, 8% CD_2H, and ~1% CDH_2.

***p*-Trideuteriomethylaniline** was prepared by dissolving 4.0 g of the above described deuterated acid in 40 ml of concentrated sulfuric acid which was overlaid with 20 ml of chloroform in a 100-ml round-bottomed flask. To this solution was added sodium azide, 1.92 g, over a period of 2.5 hr at 50–60°. The reaction was allowed to proceed an additional hour after the total amount of sodium azide had been added. It was then separated and the acid layer poured into 50 g of ice. A precipitate formed and was filtered off; the filtrate was made basic by addition of ammonium hydroxide and was extracted three times with 50-ml portions of ether which, after drying over magnesium sulfate and evaporation, afforded 2.6 g (81%) of the desired product, mp 43–45°.

***p*-Aminobenzotrifluoride** was synthesized by heating 4.16 g of *p*-trifluoromethylbenzoyl chloride (Aldrich) with 1.56 g of sodium azide in 50 ml of dry benzene for 24 hr at 55–65°. After cooling, crystalline NaCl and excess NaN_3 were filtered. The filtrate was then treated with 10 ml of concentrated sulfuric acid, added dropwise at 55–65°, and then refluxed about 8 hr until no more gas was evolved. After pouring over ice (50 g) the aqueous layer was made alkaline with concentrated NH_4OH and the amine product was extracted six times with 15-ml portions of ether. The ether was evaporated and the amine was distilled, bp 31° (4 mm). The pmr spectrum of the product showed a multiplet from 6.8 to 7.8 ppm from TMS (4 H) and a singlet at 3.9 ppm (2 H).

***o*-Toluidine, *m*-toluidine, *o*-trifluoromethylaniline, and *m*-trifluoroaniline** were commercial products (Matheson Coleman and Bell, Aldrich, and Hooker Chemical Co.). *p*-Toluidine was prepared by the same procedure as used for the deuterated compound.

All of the α-bromoacetanilides were prepared by the representative procedure which follows. *m*-Toluidine (10.0 g) was dissolved in 100 ml of ether and vigorously stirred with 50 ml of 1 *N* NaOH solution. To this two-phase system was added, dropwise, 22.2 g of bromoacetyl bromide in 100 ml of ether over a period of 1 hr with the flask immersed in an ice bath. The phases were separated and the ether layer was dried with magnesium sulfate. After evaporation the desired crude material was recrystallized from ethanol (95%) to yield 10.5 g (50%) of white needles, mp 89–90°. The physical properties of these compounds are collected in Table V. The mass spectrum of each compound showed a typical "bromine cluster" about the *m/e* corresponding to the molecular weight and an intense line at $m/e \equiv M - COCHBr$.

Table V. Physical Constants for Substituted N-Phenyl-α-bromoacetamides

Substituent	Mp, °C	Pmr spectrum, ppm[a]
None	133–134	4.1 (2 H), 7.1–7.9 (6 H)
o-CH_3	110–112	2.3 (3 H), 4.1 (2 H), 7.2–7.8 (5 H)
m-CH_3	89–90	2.3 (3 H), 4.0 (2 H), 3.2 (1 H), 6.8–7.5 (4 H)
p-CH_3	165–166	2.3 (3 H), 4.1 (2 H), 3.6 (1 H), 7.1–7.7 (4 H)
p-CD_3	159–161	4.1 (2 H), 3.1 (1 H), 7.1–7.7 (4 H)
o-CF_3	104–106 (lit.[b] 95)	4.2 (2 H), 7.4–8.7 (5 H)
m-CF_3	83–84 (lit.[b] 82)	4.0 (2 H), 7.4–7.8 (5 H)
p-CF_3	156–157 (lit.[b] 158)	4.1 (2 H), 3.0 (1 H), 7.6–8.1 (4 H)

[a] Chemical shift relative to internal TMS; solvents were either $CDCl_3$ or CD_3COCD_3. [b] K. Fukui, H. Kitano, R. Ijiri, Y. Inamoto, and T. Matsufuji, *Nippon Kagaku Zasshi*, **79**, 889 (1958); *Chem. Abstr.*, **54**, 4430b (1960).

N-Acetyl-DL-methionine was prepared by dissolving 0.1 mol (14.9 g) of DL-methionine (Pierce Chemical Co.) in 150 ml of glacial acetic acid and heating to boiling. The solution was allowed to cool to approximately 90° and 15 ml (0.15 mol) of acetic anhydride was added over a period of 15 min. After the addition the reaction mixture was boiled gently for 2 min, then cooled to 40° and the solvent evaporated *in vacuo*. The syrupy residue was treated with a 150-ml portion of water followed by evaporation of the water at 40°. This process was repeated thrice to remove all of the acetic acid. Then 75 ml of benzene was added and evaporated to dryness. The crude residue was recrystallized from ethyl acetate: yield 13.1 g (69%), mp 111–112°. The pmr spectrum of the product, in trifluoroacetic acid solution, consisted of a multiplet from 2.0 to 2.8 ppm (10 H) which was assigned to the methyl and methylene groups of the molecule. A

(20) Perhaps related to this observation is the report that the α-chymotrypsin-catalyzed hydrolysis of various ethers of N-acetyl-L-tyrosine methyl ester is sensitive to the nature of the alkyl group on the *p*-hydroxyl function: R. L. Peterson, K. W. Hubele, and C. Niemann, *Biochemistry*, **2**, 942 (1963).

(21) P. Goldman, *Science*, **164**, 1123 (1969).

(22) E. Della, *Tetrahedron Lett.*, 3347 (1966).

(23) L. Pauling, "The Chemical Bond," Cornell University Press, Ithaca, N. Y., 1967, p 152.

(24) P. M. Enriquez and J. T. Gerig, *Biochemistry*, **8**, 3156 (1969).

(25) "Kirk-Othmer Encyclopedia of Chemical Technology," 2nd ed, Vol. 9, Interscience Publishers, New York, N. Y., 1963, p 779.

(26) J. G. Atkinson, J. J. Csakvary, G. T. Herbert, and R. S. Stuart, *J. Amer. Chem. Soc.*, **90**, 498 (1968).

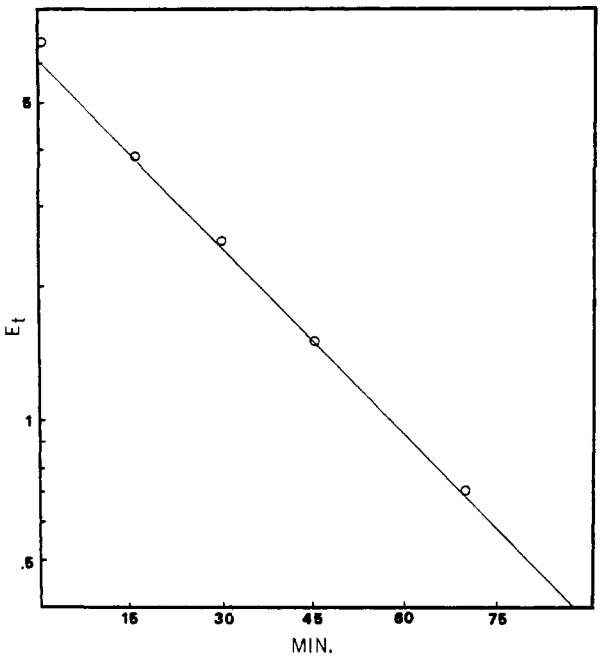


Figure 1. A typical plot of the concentration of active enzyme as a function of time. The irreversible inhibitor for this experiment was Ia; the ordinate scale is logarithmic. The concentration of α-chymotrypsin was 3.6 mg/ml and the concentration of inhibitor was 2.58 m*M*.

broad doublet at 7.7–7.8 ppm (1 H, amide) and an apparent quartet at 4.6–5.0 ppm (—C—H) were also in evidence.

N-Glutarylphenylalanine *p*-nitroanilide (GPNA) was obtained from Mann Research Laboratories.

α-Chymotrypsin (3X recrystallized) was obtained from Mann Research Laboratories or Worthington Biochemicals.

Procedures. Enzyme assays were carried out at 25.5° by measuring the initial rate of hydrolysis of a stock solution of N-glutarylphenylalanine *p*-nitroanilide (GPNA), 2×10^{-4} *M*, buffered with 0.05 *M* trihydroxymethylaminomethane at pH 7.45 with 10% DMSO as a cosolvent. Three milliliters of this stock solution was added to a uv cuvette, followed by 100 μl of the enzyme solution to be assayed (1–0.4 mg/ml). The initial rate of production of *p*-nitroaniline was measured at 410 mμ on a Gilford recording spectrophotometer at a full scale deflection of 0.200 OD unit. The extinction coefficient of *p*-nitroaniline at 410 mμ was found to be 7700 and to obey Beer's law.

All enzyme inactivations were carried out at 27.5 ± 0.5°, in water buffered at pH 6.0 with 0.1 *M* phosphate with either 10% DMSO or 10% EtOH (by volume) as a cosolvent. To 10 ml of α-chymotrypsin solution (1.8 mg/ml) was added 1.0 ml of a 1.4×10^{-2} *M* solution of α-bromoacetanilide in cosolvent. Concurrently an equivalent sample of the chymotrypsin solution was treated with the same amount of cosolvent in order to measure the loss of enzyme activity due to autolysis. This loss was significant in those reactions that required more than 2 hr for completion and the appropriate correction was applied. Aliquots (100 μl) were taken from the reaction mixture at 15-min intervals and assayed for enzymic activity remaining (E_t), as outlined above. By plotting ln [E_t] *vs.* time one obtains a straight line whose slope equals k_{obsd} (Figure 1). We have seen no evidence for irreversible inactivation taking place in the assay cell. Any reversible inhibition of the substrate reaction in the assay cell would be the same for each assay since the inhibitor concentration does not change appreciably during the inactivation reaction. The results of this reversible inhibition, then, would be to lower each point of the plot by the same amount. From the binding constants obtained, this effect is less than 8% for the best inhibitor, *p*-methyl-α-bromoacetanilide.

The reversible binding constants were determined by the following procedure. In a cuvette were placed 2.5 ml of buffer (0.05 Tris pH 7.45), 200 μl of substrate solution (either 3.1 m*M* GPNA or 1.55 m*M* GPNA), and 300 μl of ethanol with inhibitor at known concentration. The reference cell contained a sample of equivalent composition. The spectrophotometer was balanced at 0.0 OD unit and, at time zero, 100 μl of enzyme solution (3 mg/ml) was added to the reaction cell. A linear rate of increase in OD units was followed to 0.1 OD unit at 410 mμ. The binding constants are obtained by plotting the ratio of the velocity without inhibitor to the velocity with inhibitor *vs.* the inhibitor concentration. From the slope of the resulting line, K_I can be calculated (*cf.* eq 5).

A typical procedure for following the kinetics of the N-acetylmethionine model reaction is the following. Ten milliliters of a standard solution of N-acetylmethionine (7.0×10^{-2} *M*) was treated with 1 ml of the N-substituted α-bromoacetamide compound (7.05×10^{-4} *M* in ethanol) to produce a solution in which the N-acetylmethionine (6.4×10^{-2} *M*) was in 100-fold excess of the latter. The rate of production of bromide ion was followed using a Beckman bromide electrode–saturated calomel electrode system. This arrangement outputs a signal in the millivolt range that is linear in the log of the bromide ion concentration. The electrode assembly was calibrated with standardized solutions of potassium bromide.[27] The kinetic runs were done at 25.0 ± 0.5° in solutions buffered at pH 6 with 0.1 *M* phosphate with 10% ethanol (by volume) as cosolvent.

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